Infection of Human Retinal Pigment Epithelial Cells with Influenza A Viruses

Martin Michaelis, Janina Geiler, Denise Klassert, Hans Wilhelm Doerr, and Jindrich Cinatl, Jr

PURPOSE. Ocular involvement in influenza A virus diseases is common but usually limited to mild conjunctivitis. Rarely, inflammation of the choriocapillaris may result in atrophy of the retinal pigment epithelium (RPE). Primary human retinal pigment epithelial (RPE) cells were infected with seasonal (H1N1 A/New Caledonia/20/99, H3N2 A/California/7/2004) or highly pathogenic avian H5N1 (A/Thailand/1(Kan-1)/04, A/Vietnam/1203/04, A/Vietnam/1194/04) influenza strains.

METHODS. Influenza A virus replication was studied by investigation of cytopathogenic effects, immune staining for influenza A virus nucleoprotein, determination of virus titer, and electron microscopy. Apoptosis induction was examined by immune staining for activated caspase 3 and cleaved PARP. Proinflammatory gene expression was investigated by quantitative PCR.

RESULTS. H5N1 but not seasonal influenza strains replicated to high titers (>10^8 TCID50/mL; 50% tissue culture infectious dose/milliliter) in RPE cells. H5N1 infection resulted in RPE cell apoptosis that was abolished by the antiviral drug ribavirin. Pretreatment with type I interferons (interferon-a and -b) or the type II interferon, (interferon-g), inhibited H5N1 replication. Moreover, H5N1 infection induced expression of proinflammatory genes (tumor necrosis factor-a, CXCL8, CXCL10, CXCL11, and interleukin-6), which was inhibited by ribavirin in a concentration-dependent manner.

CONCLUSIONS. A novel cell type derived from the central nervous system was permissive to H5N1 influenza virus replication. This findings supports those suggesting H5N1 influenza strains to own a greater potential to spread to nonrespiratory tissues than seasonal human influenza viruses. Moreover, the data warrant the further study of the role of influenza A virus replication in retinal diseases associated with influenza A virus infections. (Invest Ophthalmol Vis Sci. 2009;50:5419–5425) DOI:10.1167/iovs.09-3752

The H5N1 influenza A viruses are considered to be potential progenitors of a new influenza pandemic.1–6 H5N1 disease is much more severe in humans than infection with seasonal human-adapted H1N1 or H3N2 influenza A virus strains, and the disease course differs in many aspects.1–6

Ocular involvement in influenza A virus is common but is usually limited to mild conjunctivitis.7–9 Cases of granulomatous iridocyclitis or diseases of the posterior segment of the eye such as inflammation of the choriocapillaris with subsequent atrophy of the retinal pigment epithelium (RPE) are much rarer.7 Ocular symptoms during influenza infection are commonly reversible, for except influenza-induced degeneration of the RPE.7 The RPE is a constituent of the blood-retina barrier that is essential for the maintenance of the immune privilege of the eye.10 The inner blood-retina barrier consists of microvascular endothelial cells and the outer blood-retina barrier consists of RPE cells.10 Notably, cases of influenza retinitis have been described in humans, especially in the context of influenza-caused central nervous system (CNS) disease.11–14 Detection of influenza virus RNA in cerebrospinal fluid (CSF) and brain tissues of influenza patients who have encephalitis/encephalopathy15,16 suggests a contribution of viral damage to these diseases. However, there is no information about influenza virus replication in the retina.

Notably, H5N1 influenza strains appear to have a greater potential to spread to nonrespiratory tissues than do seasonal “human” influenza viruses.2 H5N1 infection of humans may result in systemic infection including the CNS.17–19 In H5N1-infected mute swans, virus antigen was detected in the retina including the RPE.20 The presence of H5N1 in the retina of humans has not been investigated yet.

We established primary human RPE cell cultures to study the replication of viruses in a relevant cell culture model derived from the retina.10,21–26 In the present study, replication of avian H5N1 influenza A virus strains and seasonal human-adapted H3N2 and H1N1 influenza A virus strains were investigated in RPE cells.

MATERIALS AND METHODS

Drugs
Ribavirin (Virazole) was obtained from Valeant Pharmaceuticals GmbH (Esschborn, Germany). Interferon α-2B (Intron A) was purchased from Essex Pharma (Munich, Germany). Interferon β (Fibliferon) was received from Rentschler Biotechnologie (Laupheim, Germany). Interferon-γ1b (Imukin) was obtained from Boehringer Ingelheim (Ingelheim-am-Rhein, Germany). Interferon-β1a (Avonex) was purchased from Biogen IDEC ( Maidenhead, UK).

Virus Strains
The influenza strains A/New Caledonia/20/99 (H1N1), A/California/7/2004 (H3N2), A/Vietnam/1203/04 (H5N1), and A/Vietnam/1194/04 (H5N1) were received from the World Health Organization (WHO) Influenza Centre (National Institute for Medical Research, London). The H5N1 influenza strain A/Thailand/1(Kan-1)/04 was obtained from Pilaipan Puthavathana (Mahidol University, Bangkok, Thailand).

Virus stocks were prepared by infecting Vero cells (H5N1, African green monkey kidney; ATCC, Manassas, VA) or MDCK cells (H3N2, H1N1; ATCC), and aliquots were stored at −80°C. Virus titers were...
determined as 50% tissue culture infectious dose (TCID₅₀/ml; 50% tissue culture infectious dose/milliliter) in confluent Vero cells in 96-well microtiter plates.

Cells

RPE cells were isolated from three donors and cultured as described previously.²⁵ Briefly, the corneoscleral disc was removed first, followed by the lens and vitreous. The residual eye cup was sectioned with a longitudinal incision toward the optic nerve. Repeated rinsing with Ca²⁺ and Mg²⁺ Dulbecco’s PBS allowed prompt separation of the remaining vitreous and neural retina from the layer of RPE and permitted detachment of the choroid from the sclera. The RPE cells adhering to Bruch’s membrane on the choroidal sheets obtained were washed with PBS and treated with 0.25% trypsin-EDTA solution. Detached cells were resuspended in IMDM, supplemented with 20% FBS, and transferred to 25 cm² flasks. The homogeneity of cultured RPE cells was confirmed by positive immunostaining with mAbs to cytokeratins (Pan) and to cellular retinaldehyde binding protein (mAbs were donated by John Saari, Department of Ophthalmology, University of Washington, School of Medicine, Seattle, WA).²⁷ The cell cultures used in this study were routinely tested for mycoplasma and were not used in the experiments later than passage 3.

A549 cells (human lung carcinoma; ATCC: CCL-185) were grown at 37°C in minimal essential medium (MEM) supplemented with 10% FBS, 100 IU/mL of penicillin, and 100 μg/mL streptomycin.

Immune Staining

Cells were fixed with 40:60 acetonemethanol for 15 minutes. For detection of influenza A nucleoprotein, the monoclonal antibody MsX Influenza A (Chemicon, Hoffheim, Germany) was used as the primary antibody. Activated caspase-3 and the 85-kDa fragment of cleaved poly(ADP-ribose) polymerase (PARP) were detected by the use of the following primary antibodies: caspase-3 (active; R&D Systems, Wiesbaden, Germany) and PARP p85-fragment (Promega, Mannheim, Germany). Biotin-conjugated secondary monoclonal antibodies were used and visualization was performed with streptavidin-peroxidase complex with 3-amino-9-ethylcarbazole as the substrate.

Electron Microscopy

Cells were processed for ultrastructural analysis, as described previously.²² Briefly, cells were pelleted and fixed with 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in Durupan-Epon. Thin sections were contrasted with uranyl acetate and lead citrate and viewed with an electron microscope (JEM, 2000 CX, JEOL, Arishima, Japan).

Cytopathogenic Effect Reduction Assay

The cytopathogenic effect (CPE) reduction assay was performed as described before.²⁵ Confluent RPE monolayer grown in 96-well microtiter plates were infected with influenza A strains in the presence or absence of the investigated agents. The virus-induced CPE was recorded at 48 hours post infection (p.i.) with an inverted light microscope.

Real-Time PCR

Total RNA was isolated from cell cultures (TRI reagent; Sigma-Aldrich, Munich, Germany). Gene expression on the mRNA level was detected (TaqMan Gene Expression Assays; Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s instruction.

Real time PCR for H5 was performed according to published methods.²³ The following primers were used: sense 5’ acg tat gag tac ccc cag tat tca g 3’; antisense 5’ aga cca gcc acc aag gtc gc 3’; probe 6-FAM-tca aca gtt ggc agc ttc cta gca-TAMRA.

RESULTS

Influenza A Virus Replication in RPE Cells

In RPE cells infected with the H1N1 influenza strain A/New Caledonia/20/99 (MOI 0.01), titers remained in the range of input virus levels. Infection of RPE cells with the H3N2 strain A/California/7/2004 (MOI 0.01) resulted in a slight titer increase (10-fold) over the input level. In sharp contrast, H5N1 influenza strain A/Thailand/1(Kan-1)/04 (MOI 0.01) infection of RPE cells resulted in virus titers >10⁵ TCID₅₀/mL 48 hours p.i. (Fig. 1A). Virus titers similar to those determined for H5N1 A/Thailand/1(Kan-1)/04 were detected in RPE cells infected with the H5N1 strains A/Vietnam/1203/04 and A/Vietnam/1194/04 48 hours p.i. (MOI 0.01; Fig. 1B).

Immune staining for influenza A nucleoprotein revealed infection of all H5N1 A/Thailand/1(Kan-1)/04 (MOI 0.01)–infected RPE cells, whereas only a very few cells were found to be infected after infection with H1N1 (MOI 0.01; Fig. 1C). Cells were infected at MOI 1 (Table 1), to investigate whether reduced replication of seasonal influenza A virus strains may be caused by low (H3N2) or no (H1N1) ability of seasonal influenza A virus strains to infect RPE cells; 100% of the cells stained positively for influenza A nucleoprotein at 48 hours after infection with all viruses except H1N1 (84.42 ± 6.22). This result suggests that seasonal influenza A virus strains are able to infect RPE cells but cannot complete full replication cycles.

The described experiments were performed in the absence of trypsin. Addition of trypsin did not significantly alter results. RPE cultures derived from the retina of two other donors showed similar sensitivity to influenza A virus infection (data not shown).

Apoptosis Induction by H5N1 Influenza Virus Infection in RPE Cells

Apoptosis induction is thought to play a role in H5N1 pathogenesis.²⁴ H5N1 viruses have been shown to induce apoptosis in different cell types including pneumocytes, immune cells, and CNS cells.¹⁰ ²⁸–²⁹ H5N1-induced apoptosis was detected in RPE cells by immune staining. The number of apoptotic cells was determined by immune staining for activated caspase 3 or cleaved PARP by counting the positively stained cells in the central fields of three different wells (3.7 mm²) of 96-well plates at 50× magnification. Cells that stained positively for activated caspase-3 or cleaved PARP 48 hours p.i. with the H5N1 virus strain A/Thailand/1(Kan-1)/04 (MOI 0.01) were quantified relative to noninfected cells. H5N1 (A/Thailand/1(Kan-1)/04) infection resulted in a substantial increase in the number of apoptotic cells (Fig. 2).

Since it is consistently active against a broad number of influenza viruses,³³ ribavirin is commonly used for inhibition of influenza replication in experimental settings, although it is not clinically approved for the treatment of influenza. The ribavirin concentration that inhibited 50% of H5N1 A/Thailand/1(Kan-1)/04 virus-induced CPE formation (IC₅₀) was 4.8 ± 2.1 μg/mL. Ribavirin 20 μg/mL treatment suppressed H5N1-induced apoptosis in RPE cells (Fig. 2) indicating that virus replication is necessary for apoptosis induction. Similar results were obtained for the H5N1 strain A/Vietnam/1203/04 (data not shown).

Electron microscopic examination of H5N1 A/Thailand/1(Kan-1)/04-infected RPE cells revealed that apoptosis is induced in H5N1 virus producing RPE cells (Fig. 2) as indicated by membrane blebbing and nuclear fragmentation.
Influence of Interferons on H5N1 A/Thailand/1(Kan-1)/04 Infection of RPE Cells

Interferons were shown to be active against influenza virus infection and are discussed as potential anti-influenza drugs in case of an H5N1 pandemic. Therefore, antiviral effects of type I interferon-α and -β or type II interferon-γ were studied in RPE cells. Pretreatment for 24 hours with all three interferons protected RPE cells from H5N1 A/Thailand/1(Kan-1)/04 virus-induced CPE in a concentration-dependent manner (Fig. 3A). The lowest IC₅₀s were obtained for interferon-α (43.0 ± 26.9; Fiblaferon; Rentschler Biotechnologie) (Table 2). Effects comparable to those detected for interferon-β were induced by interferon-β1a (Avonex; Biogen IDEC) (data not shown).

A549 lung cancer cells resemble human alveolar type II epithelial cells. IC₅₀s for the type I interferons were lower in A549 cells, whereas IC₅₀s for interferon-γ were higher in A549 cells than in RPE cells (Fig. 3; Table 2).

Influence of H5N1 A/Thailand/1(Kan-1)/04 Infection on Proinflammatory Gene Expression in RPE Cells

Expression of the proinflammatory genes tumor necrosis factor α (TNF-α), CXCL8, CXCL10, CXCL11, or interleukin-6 (IL-6) was detected in H5N1 A/Thailand/1(Kan-1)/04, A/Vietnam/1203/04, A/Vietnam/1194/04, H3N2 A/California/7/2004, or H1N1 A/New Caledonia/20/99-infected RPE cells. Expression of all five genes was substantially increased in H5N1-infected cells (although the individual gene expression strongly differed) but only moderately (H3N2) or was not (H1N1) affected by seasonal influenza virus strains (Fig. 4). Ribavirin inhibited expression of the influenza H5 gene (indicating its influence on H5N1 replication) and of proinflammatory genes in H5N1 A/Thailand/1(Kan-1)/04-infected RPE cells in a dose-dependent manner (Fig. 5).

**TABLE 1.** Fraction of Influenza A Virus-Infected RPE That Cells Stained Positively for Influenza A Virus Nucleoprotein 48 hours Post Infection

<table>
<thead>
<tr>
<th>Influenza A Nucleoprotein-Positive Cells (%)</th>
<th>MOI 0.01</th>
<th>MOI 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/New Caledonia/20/99 (H1N1)</td>
<td>&lt;1</td>
<td>84.4 ± 6.2*</td>
</tr>
<tr>
<td>A/California/7/2004 (H3N2)</td>
<td>2.6 ± 2.1</td>
<td>100</td>
</tr>
<tr>
<td>A/Thailand/1(Kan-1)/04 (H5N1)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A/Vietnam/1203/04(H5N1)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A/Vietnam/1194/04(H5N1)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Mean ± SD (n = 3).
DISCUSSION

This is the first report in which influenza A virus replication in human RPE cells was investigated. RPE cells represent a neuronal cell type that may be relevant in influenza A virus-induced retinal pathogenesis.11–14,20 Influenza A virus replication was investigated in primary RPE cell cultures. The seasonal human-adapted influenza A strains A/New Caledonia/20/99 (H1N1) and A/California/7/2004 (H3N2) did not or only very slightly replicated in this cell type. In contrast, the highly pathogenic H5N1 avian influenza strains A/Thailand/1(Kan-1)/04, A/Vietnam/1203/04, and A/Vietnam/1194/04 replicated to high titers (>10^8 TCID50/mL) in RPE cells.

RPE cultures were sensitive to infection (MOI 1) with seasonal influenza A viruses (80–100% of infected cells) or H5N1 viruses (100% of infected cells). These findings do not reflect the differences detected in the production of infectious virus.

Therefore, seasonal influenza A viruses and H5N1 viruses obviously possess different abilities to perform complete replication cycles in RPE cells. The observation that H5N1 viruses have a greater potential to replicate in RPE cells than do seasonal influenza A strains is in accordance with findings that suggest H5N1 influenza strains to have a greater potential to replicate in nonrespiratory tissues, including the CNS, than seasonal influenza viruses.2,17–19 Of note, influenza retinitis has been correlated to influenza CNS disease.11–14 Therefore, the ability to infect RPE cells may be limited to a (small) subset of seasonal CNS-tropic influenza A virus strains. Taking into account the higher CNS tropism of H5N1 strains, the spread of H5N1 infection in humans may result in an increase in cases of influenza A virus retinitis.

The permissiveness of RPE cells to influenza A virus infection correlated with the virus’ ability to induce apoptosis. Infection (MOI 0.01) with H5N1 resulted in substantial apoptosis in RPE cells. (A) Relative number of RPE cells infected with the H5N1 virus strain A/Thailand/1(Kan-1)/04 (MOI 0.01) stained positively for activated caspase-3 in absence or presence of ribavirin (20 μg/mL) 48 hours p.i. compared with noninfected (Mock) cells. (B) Relative number of RPE cells infected with the H5N1 virus strain A/Thailand/1(Kan-1)/04 (MOI 0.01) stained positively for the 85-kDa fragment of cleaved poly(ADP-ribose) polymerase (PARP) in the absence or presence of ribavirin (20 μg/mL) 48 hours p.i. compared with noninfected (Mock) cells. (C) Electron micrographs of noninfected (Mock) or H5N1 virus strain A/Thailand/1(Kan-1)/04 (MOI 0.01)–infected RPE cells 48 hours p.i. Produced virus particles are shown in the inset at a higher magnification. The infected cell shows clear signs of apoptosis including membrane blebbing and nuclear fragmentation. Data are the mean ± SD (n = 3). Scale bar: (C) 1 μm; (inset), 0.1 μm.
Interferon-

findings by other groups challenged these conclusions.35–37,44

MOI 0.1)-Infected RPE or A549 Cells Detected 48 Hours p.i.

against CPE Formation in H5N1 A/Thailand/1(Kan-1)/04 Strain

dependent pathway in human airway epithelial cells.29,39 Of

over, H5N1 infection induced apoptosis mainly via the caspase-

**FIGURE 4.** Expression of the proinflammatory genes *TNF*-α, *CXCL8*, *CXCL10, CXCL11*, or IL-6 in retinal pigment epithelial cells infected with A/Thailand/1(Kan-1)/04 (H5N1 KAN), A/Thailand/1(Kan-1)/04 (H5N1 Viet), A/California/7/2004 (H3N2), or A/New Caledonia/20/99 (H1N1) (MOI 0.01) as indicated by quantitative real-time PCR 24 hours p.i. Data are the mean ± SD (n = 3).

interferons interfered with H5N1 replication in RPE cells. Moreover, A549 cells resembling human alveolar type II epithelial cells were also protected by type I and II interferons from H5N1 infection. These findings favor a potential role of interferons as treatment options in the case of H5N1 pandemic.

Virus infection of RPE cells was previously shown to result in an interferon response that may limit virus replication.52 The NS1 proteins of H5N1 viruses have been reported to better inhibit a cellular interferon response than do NS1 proteins of seasonal influenza A viruses.12,43 Therefore, differences in the ability of H5N1 NS1 proteins and NS1 proteins of seasonal influenza strains may contribute to the different influenza A virus replication kinetics in RPE cells observed herein. Addition of interferons after infection resulted in strongly decreased antiviral effects of interferons (data not shown). Moreover, infection of RPE cells with H1N1-induced MxA expression and phosphorylation of STAT1 and -2 indicating induction of interferon signaling, whereas RPE cell infection with H5N1 did not (data not shown). This result may indicate that H5N1 viruses are very efficient in antagonizing the interferon response once an RPE cell is infected, although they remain sensitive to pretreatment with interferons.

Elevated levels of different cytokines/chemokines (hypercytokinemia), detected in the blood of humans infected with H5N1 strains, were suggested to contribute to the pathogenesis of H5N1 disease. Since serum cytokine/chemokine levels do not necessarily reflect the local production of these regulatory proteins in the lungs or other infected tissues, it is important to study the effects of H5N1 on cytokine/chemokine expression in specific cell types of infected tissues.6 Proinflammatory gene expression may be altered in cells from immune-privileged sites, such as RPE cells.10 Elevated levels of TNF-α, CXCL8 (also known as interleukin 8, IL-8), CXCL10 (also known as interferon γ-inducible protein 10, IP-10), and IL-6 were detected in the plasma of H5N1-infected humans.6 Influenza A virus infection of human blood dendritic cells has been shown to trigger CXCL11 (interferon γ-inducible protein 9, IP-9) production by these cells.65 H5N1 infection of RPE cells enhanced expression of all five cytokines. Expression patterns of proinflammatory molecules differ between macrophages and airway epithelial cells, as indicated by experiments using cultured cells as well as by pathologic examination of the lung of a patient
who died of H5N1 influenza. Proinflammatory gene expression in H5N1-infected RPE cells more closely resembles that of infected macrophages. For example, TNF-α, IL-8, and IL-6 were found to be upregulated in H5N1-infected RPE cells and macrophages but not in airway epithelial cells. Ribavirin inhibited upregulation of all five investigated cytokines in RPE cells in a concentration-dependent manner. These data suggest that H5N1 infection of RPE cells results in a proinflammatory response that can at least in part be impeded by antiviral therapy.

In conclusion, we showed that primary human RPE cells are permissive to H5N1 influenza virus replication. Therefore, RPE cells represent a model for the study of H5N1 influenza virus biology in cells derived from the CNS. Moreover, our data warrant further study of the role of influenza virus replication in retinal diseases associated with influenza A virus infections.

Acknowledgments
The authors thank Gesa Meincke, Kerstin Euler, Lena Stegmann, and Elena Brandi-Barbarito for technical assistance.

References


